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Effect of Calcium in Assay Medium on *D* Value of *Bacillus stearothermophilus* ATCC 7953 Spores

KOICHI SASAKI,[1]* HIDEHARU SHINTANI,[2] JUNPEI ITOH,[3] TAKUJI KAMOGAWA,[1] AND YOUSEI KAJIHARA[4]

Quality Control Department, Misato Plant, Eisai Co., Ltd., Misato-Machi, Kodama-Gun, Saitama 367-0198,[1] Division of Medical Devices, National Institute of Health Sciences, Setagaya-ku, Tokyo 158-0098,[2] PPM Manufacturing, Fukushima Plant, Nippon Becton Dickinson Company, Ltd., Tsuchifune, Fukushima City, Fukushima 960-2152,[3] and Life Tech Division, Nihon Pharmaceutical Co., Ltd., Sumiyoshi-cho, Izumisano City, Osaka 598-8558,[4] Japan

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The *D* value of commercial biological indicator spore strips using *Bacillus stearothermophilus* ATCC 7953 was increased by higher calcium concentrations in assay media. The calcium concentration in assay media varied among the manufacturers. The calcium concentration in assay media is an important factor to consider to minimize the variation of *D* value.

The effectiveness of sterilization is examined using biological indicators (BI). Use of a BI provides a means to directly examine death of microorganisms in the sterilization process. Therefore, determination of the resistance of BI against the sterilization method (*D* value) is an extremely important parameter for evaluation of the effectiveness of the sterilization process and equipment. In autoclaving, *Bacillus stearothermophilus* ATCC 7953 spores are normally used as BI. Generally, the *D* value of BI is determined by using a soybean casein digest agar medium (SCDA) (15.0 g of casein digest peptone per liter, 5.0 g of soybean digest peptone per liter, 5.0 g of NaCl per liter, 15.0 g of agar per liter [pH 7.1 to 7.5]) for the survivor curve method but soybean casein digest broth medium (SCDB) (17.0 g of casein digest peptone per liter, 3.0 g of soybean digest peptone per liter, 5.0 g of NaCl per liter, 2.5 g of K_2HPO_4 per liter, 2.5 g of glucose per liter [pH 7.1 to 7.5]) for the fraction negative method. The *D* value of *B. stearothermophilus* ATCC 7953 spores at 121°C was significantly different when it was determined by using media with the same composition made by different manufacturers (2). However, the reason why the *D* value varies among media of different manufacturers is not known, and resolution of this problem is acutely needed to advance validation for scientific assurance of the sterilization process. We studied the relationship between the calcium concentration of the medium and the growth of *B. stearothermophilus* ATCC 7953 spores, because calcium is known to affect the heat resistance and/or germination of bacterial spores (1, 3–10).

Abbreviations. BBL, Becton Dickinson Microbiological Systems; DAIGO, Nihon Pharmaceutical Co., Ltd.; BIER, biological indicator evaluation resistometer; NAmSA, North American Science Associates, Inc., ISO, International Organization for Standardization; BAPTA, *O*,*O*′-bis(2-aminophenyl)-ethyleneglycol-*N*,*N*,*N*′,*N*′-tetraacetic acid.

Relationship between the *D* value and the calcium concentration in the assay medium. The calcium concentration in the media was determined by using an atomic absorption spectrophotometer (AAnalyst 800; Perkin-Elmer, Norwalk, Conn.). The absorbance was measured at 422.7 nm using a calcium hollow-cathode lamp as the radiation source and a nitrous oxide-acetylene flame. The ashing procedure was performed by the method described in *United States Pharmacopeia* (9a). The calcium concentrations of the media are shown in Table 1.

(i) SCDA. Commercial BI (paper strip type SPORTROL STS-05; lot no. S53003) was purchased from NAmSA. The BI manufacturer's specifications were the initial population of 1.8×10^5 spores per strip and the *D* value of 1.9 min (at 121°C). Commercial SCDA was obtained from four different manufacturers (Difco Laboratories [Detroit, Mich.], BBL, Oxoid Ltd. [Basingstoke, England], and DAIGO). Three separate lots of SCDA from each manufacturer were used (Difco lot no. 58057JD, 73273JF, and 100020JB; BBL lot no. E8DFCF, F8DFFV, and J9DHAT; Oxoid lot no. 166 56368, 009 57341, and 219 56605; DAIGO lot no. S105, S109, and S121). The *D* values were determined using the survivor curve method by standard plating techniques (7a). Six replicate strips were exposed at various time intervals to saturated steam at 121°C in BIER (Joslyn Sterilizer Corporation). After heating, BI was transferred immediately to sterile purified water. BI was homogenized to obtain spore suspensions, if necessary, by serial dilution with sterile purified water. One milliliter (each) of these spore suspensions was placed in petri dishes, into which 20 ml of sterilized assay medium was poured. After solidification, they were cultured at 57.5 ± 1°C for 72 h. The logarithmic number of survivors was plotted against exposure time, and the optimal line was determined by regression analysis using the least-squares method. The reciprocal of the slope of the line obtained was calculated, and the *D* value was determined.

The calcium concentration in the prepared SCDA was not different among lots of SCDA from the same manufacturer but differed significantly among products from different manufacturers at 1% level ($\alpha = 0.01$). The four manufacturers could be divided into two groups according to the calcium concentration, which showed significant differences at 1% level ($\alpha = 0.01$). Difco and DAIGO belonged to a high-calcium group (mean, 1.41 mM). BBL and Oxoid belonged to a low-calcium group (mean, 0.48 mM). The mean calcium concentrations of the two groups were 3:1 with a difference of 0.93 mM.

A correlation ($r = 0.921$) was observed between the calcium concentration of SCDA and the *D* value (Fig. 1A). The *D* value was highest at 3.27 min when it was determined using SCDA

* Corresponding author. Mailing address: Quality Control Department, Misato Plant, Eisai Co., Ltd., 950, Hiroki, Misato-Machi, Kodama-Gun, Saitama 367-0198, Japan. Phone: 81-495-76-4365. Fax: 81-495-76-3942. E-mail: k-sasaki@hhc.eisai.co.jp.

TABLE 1. Calcium concentration in assay media from different manufacturers

Medium	Manufacturer	Trade name	Lot no.	Calcium concn in:	
				Powder (% [wt/wt])	Prepared medium[a] (mM)
SCDA	Difco	Tryptic Soy Agar	58057JD	0.134	1.34
			73273JF	0.162	1.63
			100020JB	0.127	1.27
	BBL	Trypticase Soy Agar	E8DFCF	0.068	0.68
			F8DFFV	0.021	0.21
			J9DHAT	0.039	0.39
	Oxoid	Tryptone Soya Agar	166 56368	0.036	0.36
			009 57341	0.073	0.73
			219 56605	0.051	0.51
	DAIGO	Soybean Casein Digest Agar	S105	0.122	1.22
			S109	0.136	1.37
			S121	0.165	1.65
SCDB	Difco	Tryptic Soy Broth	104075JB	0.030	0.22
			66933JB	0.032	0.24
			72198JC	0.032	0.24
	BBL	Trypticase Soy Broth	A8DENZ	0.031	0.23
			D8DFAZ	0.052	0.39
			G8DFHH	0.044	0.33
	Oxoid	Tryptone Soya Broth	018 57329	0.074	0.55
			073 55886	0.050	0.37
			188 56515	0.046	0.34
	DAIGO	Soybean Casein Digest Broth	S107	0.140	1.05
			S128	0.176	1.32
			S130	0.129	0.97

[a] SCDA (40 g/liter) and SCDB (30 g/liter).

from DAIGO (lot no. S121) and lowest at 1.73 min when it was determined using SCDA from Oxoid (lot no. 166 56368), with a difference of 1.54 min. The *D* values obtained showed no significant difference among the lots of the same manufacturer but differed significantly among the manufacturers at the 1% level ($\alpha = 0.01$). These results strongly suggest that the concentration of calcium in SCDA is largely responsible for the variation of the *D* value of BI.

(ii) SCDB. Commercial BI (paper strip type SPORTROL STS-05; NAmSA lot no. S62705) was used. The BI manufacturer's specifications were the initial population of 1.9×10^5 spores per strip and the *D* value of 1.5 min (at 121°C). Com-

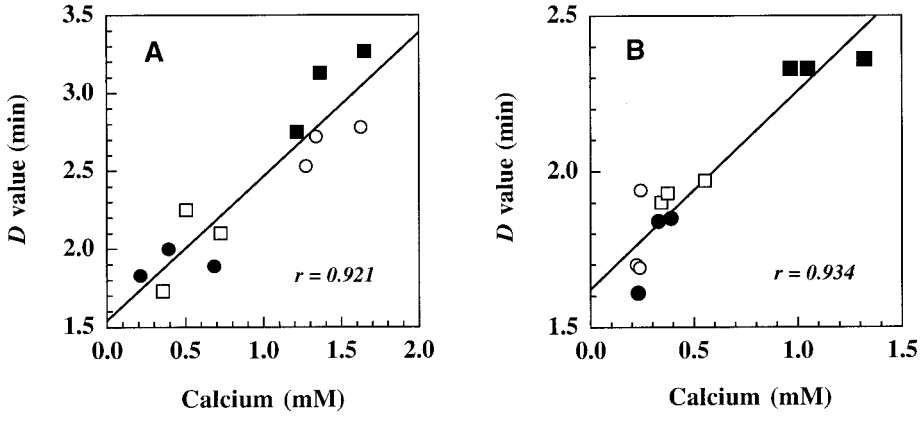

FIG. 1. Relationship between the *D* value of *B. stearothermophilus* ATCC 7953 spores and the calcium concentration in assay medium. Commercial SCDA (A) and SCDB (B) were used. Each point represents the mean of three independent experiments. Symbols: ○, Difco; ●, BBL; □, Oxoid; ■, DAIGO. The calcium concentration is the value in prepared medium.

mercial SCDB was obtained from four different manufacturers (Difco, BBL, Oxoid, and DAIGO). Three separate lots of SCDB from each manufacturer were used (Difco lot no. 104075JB, 66933JB, and 72198JC; BBL lot no. A8DENZ, D8DFAZ, and G8DFHH; Oxoid lot no. 018 57329, 073 55886, and 188 56515; DAIGO lot no. S107, S128, and S130). The *D* values were determined using the fraction negative method. The groups of 50 replicate strips were exposed to saturated steam at 121°C in a BIER. After heating, each strip of BI was immediately transferred to a test tube that contained 10 ml of SCDB. These test tubes were cultured at 57.5 ± 1°C for 7 days. After incubation, the number of strips negative for growth was used to calculate the *D* value according to the method of limited Stambo-Murphey-Cochran procedure (7b).

The calcium concentrations in the prepared SCDB were also not significantly different among lots of the same manufacturer but showed significant differences among products of different manufacturers at the 1% level ($\alpha = 0.01$). The manufacturers could be divided into two groups: DAIGO in one group and the other three manufacturers (Difco, BBL, and Oxoid) in another group. The calcium concentration was high in DAIGO (mean, 1.11 mM) but low in the other group (mean, 0.32 mM) with a significant difference at 1% ($\alpha = 0.01$). The mean calcium concentration in DAIGO was 3.4 times higher than the mean of the other three manufacturers, with a difference of 0.79 mM.

A correlation ($r = 0.934$) was observed between the calcium concentration of SCDB and the *D* value (Fig. 1B). The *D* value was highest at 2.36 min when it was determined by using SCDB from DAIGO (lot no. S128) but lowest at 1.61 min when it was determined by using SCDB from BBL (lot no. A8DENZ), with a difference of 0.75 min. The *D* values obtained showed no significant difference among the lots of a manufacturer but differed significantly among products of different manufacturers at the 1% level ($\alpha = 0.01$). These results strongly suggest that calcium in SCDB is largely responsible for the variation of the *D* value of BI.

Effect of calcium on the *D* value. A 10 mM calcium solution was prepared by dissolving $CaCl_2 \cdot 2H_2O$ (Kanto Chemical Co., Inc., lot no. 603E1423) with purified water and used after sterilization by filtration (pore size, 0.22 μm). The BI were heated at 121°C with a BIER. The initial population of BI was determined after heat activation at 100°C for 15 min.

(i) SCDA. Commercial BI (paper strip type SPORTROL STS-05; NAmSA lot no. S53003) was used. SCDA with different total calcium concentrations (0.5, 1.0, 1.5, 2.0, and 2.5 mM) were prepared by adding this calcium solution to sterilized SCDA (BBL lot no. F8DFFV). Changes in the logarithmic mean of the number of surviving spores in calcium-supplemented SCDA were plotted in Fig. 2. The number of surviving spores in all heating conditions was increased significantly by adding calcium to the original SCDA (calcium concentration of 0.21 mM) at the 5% level ($\alpha = 0.05$). The effect of adding calcium on the number of surviving spores was more pronounced as the heating time was increased (Fig. 2). This result suggests that spores are more dependent on calcium for germination or outgrowth, as a higher percentage of spores are heat injured. Therefore, calcium is considered to be involved in the repair of heat-injured spores.

The change in the *D* value was examined with calcium-supplemented SCDA (Fig. 3A). The *D* value increased with the calcium concentration and nearly reached a plateau at 3.07 min at calcium concentrations of 2.0 mM or above from the value in the original SCDA of 1.87 min. This result suggests that the optimal calcium concentration in the assay medium is required for maximum and consistent *D* value determination.

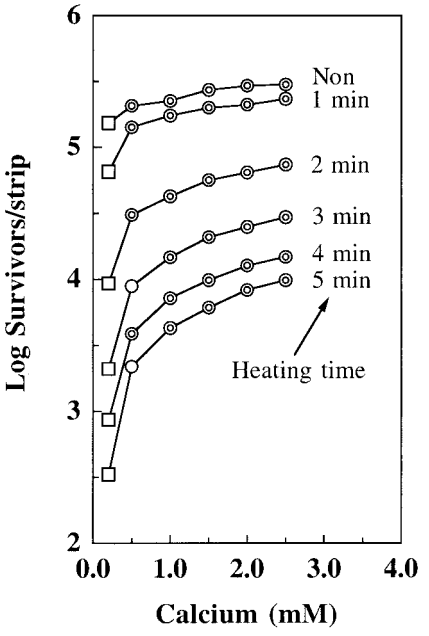

FIG. 2. Effect of calcium on the logarithmic mean of the number of surviving spores. The calcium concentration is the value in prepared medium. Each point represents the mean of three independent experiments. Statistical analysis of data was performed for the logarithmic mean of surviving spore counts between original SCDA (□) (calcium concentration, 0.21 mM) and calcium-supplemented SCDA. Statistical significance of differences at the 5% level ($\alpha = 0.05$) (○) and the 1% level ($\alpha = 0.01$) (◎) is indicated. Non, heat activation only. Heating times involved a temperature of 121°C.

A calcium concentration of about 2.0 mM was shown to be necessary to obtain consistent *D* values using SCDA.

(ii) SCDB. SCDB requires a more complex experimental design than SCDA, because SCDB contains K_2HPO_4. Calcium is generally considered to bind with phosphoric acid and to form insoluble calcium phosphate in the autoclaving process, rendering it unavailable to microorganisms. Therefore, this assumption is in conflict with the fact that there was a correlation between the calcium concentration in the medium and the *D* value also in SCDB (Fig. 1B). To answer this question, we conducted two experiments with SCDB, i.e., the *D* value was determined for SCDB with added calcium before autoclaving and for SCDB supplemented with calcium after autoclaving. SCDB media with different total calcium concentrations (0.5, 0.75, 1.0, 1.25, 1.75, 2.25, 2.75, and 3.25 mM) were also prepared by adding the same calcium solution to sterilized SCDB (BBL lot no. A8DENZ). Other SCDB media were prepared by adding $CaCl_2 \cdot 2H_2O$ to SCDB powder, dissolving the powder, and sterilizing the solution by heating. Commercial BI (paper strip type SPORTROL STS-05; NAmSA lot no. S62705) was used.

In this experiment, the solution became turbid when calcium solution was added to SCDB at high calcium concentrations. This milky precipitate was washed and centrifuged. After the milky precipitate was dried, the elements were analyzed by the energy-dispersive X-ray spectroscopy system (scanning electron microscopy [JSM-5400; JEOL] and an energy-dispersive X-ray spectrometer [PV9800; EDAX]). The P/Ca ratio of milky precipitate was 1.0. This result suggested that the precipitates obtained from the turbid SCDB were $CaHPO_4$.

The *D* values determined by the two methods agreed at calcium concentrations of 1.25 mM or less, and the *D* value increased with the calcium concentration in both experiments

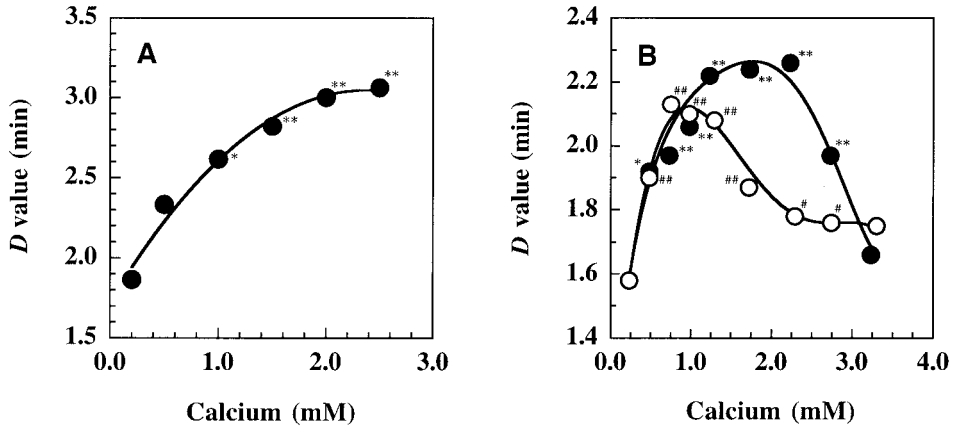

FIG. 3. Effect of calcium on the *D* value of *B. stearothermophilus* ATCC 7953 spores. The calcium concentration was changed by the addition of $CaCl_2$ solution to commercial (original) SCDA (A) and SCDB (B) after autoclaving (●) or before autoclaving (○). The calcium concentration is the value in prepared medium. Each point represents the mean of three independent experiments. Statistical analysis of data was performed for the *D* value between the original medium and calcium-supplemented medium. Statistical significance of differences at the 5% level ($\alpha = 0.05$) (*, #) and at the 1% level ($\alpha = 0.01$) (**, ##) is indicated.

(Fig. 3B). However, at calcium concentrations exceeding 1.25 mM, the *D* value decreased in SCDB with added calcium before autoclaving, because of the formation of $CaHPO_4$ by heating.

On the other hand, when calcium was added after autoclaving, the *D* value increased to a calcium concentration of 2.25 mM, a value 1.43 times the value obtained in the original SCDB. However, it decreased at calcium concentrations above 2.25 mM, also because of the formation of $CaHPO_4$. From these results, it may be concluded that within certain ranges, the *D* value is correlated with the calcium concentration. However, why $CaHPO_4$ is not formed at a low calcium concentrations is unknown at present.

The important role played by calcium in the assay medium for the determination of accurate *D* values was further demonstrated by the supplementation experiments.

Effects of calcium on germination and/or outgrowth. To test whether calcium is an indispensable factor in germination and/or outgrowth of spores of *B. stearothermophilus*, we used a specific Ca^{2+} chelating agent, BAPTA (Dojindo Laboratories lot no. GN116) (tetrapotassium salt, hydrate). Commercial BI (paper strip type SPORTROL STS-05; NAmSA lot no. S62705) was used. BAPTA was added to a final concentration of 0.25 mM to 10 ml of SCDB (BBL lot no. A8DENZ). To these media, BI that was simply activated by heating (100°C for 15 min) or BI that was heated at 121°C for 10 min with BIER was added. Fifty strips of BI were used under each condition. Two sets of samples were prepared for each condition. The same procedure was performed with media not containing BAPTA as controls. BI was cultured in these media at 57.5 ± 1°C for 7 days. After culturing, a calcium solution was added to the controls and one of the two sets of the media at 0.25 mM,

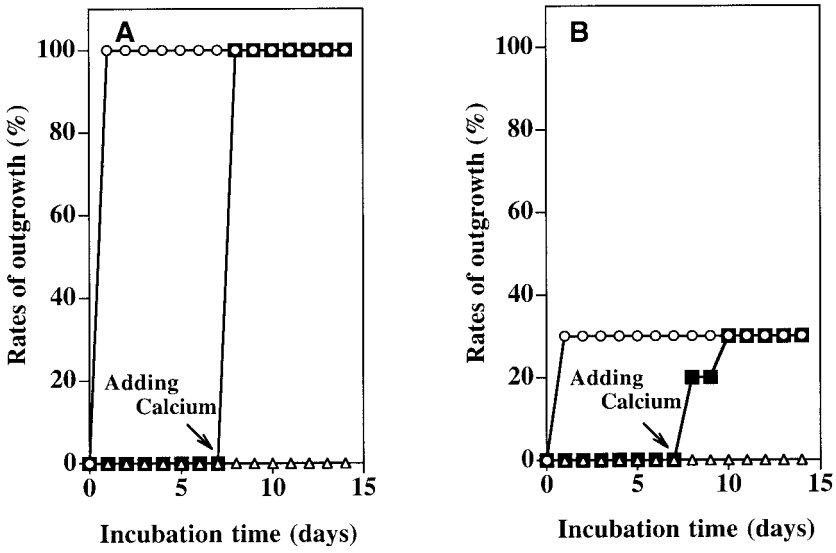

FIG. 4. Effect of calcium on the outgrowth rate of *B. stearothermophilus* ATCC 7953 spores. The BI were simply activated by heating (A) or were heated at 121°C for 10 min (B). The outgrowth rates were the percentages of positive BI strips/tested BI strips. Each point represents the mean of three independent experiments. Symbols: ○, control (SCDB); ■, added $CaCl_2$ solution to SCDB containing 0.25 mM BAPTA after 7 days (final calcium concentration, 0.25 mM); △, SCDB containing 0.25 mM BAPTA.

and BI was cultured for 7 more days. Each culture was observed daily for the growth of BI after germination.

All spores that were simply heat activated showed outgrowth after 1 day of culturing in BAPTA-free SCDB (Fig. 4A). However, no outgrowth was noted in heat-activated spores in BAPTA-supplemented SCDB, even after 7 days of culturing (Fig. 4A). When calcium was added on day 7 of culturing, outgrowth occurred in 100% of the spores, even in BAPTA-supplemented SCDB (Fig. 4A). On the other hand, no outgrowth was observed in calcium-free SCDB even after 14 days of culturing (Fig. 4A). When the spores were treated at 121°C for 10 min, 30% of the spores showed outgrowth in BAPTA-free SCDB (Fig. 4B). However, in BAPTA-supplemented SCDB, no outgrowth was noted even after 7 days of culturing (Fig. 4B). When calcium was added on day 7 of culturing, outgrowth was noted in 20% of the spores on the next day and in 30% of the spores after 3 days (Fig. 4B). In contrast, no outgrowth was observed in calcium-free SCDB even after culturing for 14 days (Fig. 4B). From these results, it appears certain that calcium is an indispensable factor in the germination and/or outgrowth of spores regardless of heat injury. Whether calcium is needed for germination or outgrowth could not be determined.

In this study we determined that calcium concentration in assay media plays an important role in the *D* value. We concluded that more-consistent determination of the *D* value of *B. stearothermophilus* ATCC 7953 spores becomes possible when the calcium concentration of the medium is controlled at an appropriate level. Although calcium was shown to be important for the germination or outgrowth of *B. stearothermophilus*, its mode of action has not been elucidated. It remains to be determined at which stage of the germination calcium is involved. It is also necessary to examine whether the importance of calcium in the assay media is limited to *B. stearothermophilus* ATCC 7953 spores or whether it applies to all bacterial spores. Research concerning these points is expected to lead to the establishment of conditions that allow more consistent determination of the *D* value. Since BI of different lots was used in this study, comparison between the survivor curve method using agar media and the fraction negative method using liquid media was not possible. In the future, we will study whether there is a difference between the two methods and attempt to develop suitable media for more-consistent determination of the *D* value of *B. stearothermophilus* ATCC 7953 spores.

The official document (7c) requires that the *D* value of BI be within 0.5 min of the value declared by the manufacturer. However, this study showed that this requirement might not be met if assay media with different calcium concentrations are used. The *D* values determined in this study were different from the values determined by BI manufacturers because different manufacturers use different media of their choice. This particular point is important when users of BI select assay media for the determination of the *D* value.

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